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(54) Title: PURIFICATION AND DETECTION PROCESSES USING REVERSIBLE AFFINITY ELECTROPHORESIS

(57) Abstract

An affinity electrophoresis process is described, in which the direction of electrophoresis is varied in a cyclical manner while synchronously changing one or more property of the electrophoretic medium between two states, said states being characterized as favoring or disfavoring specific reversible binding of sample analytes to affinity ligands which are immobilized within the medium. The resulting process enables extremely efficient and convenient separation of the specific analytes for detection or purification, using simple materials and apparatus.

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PURIFICATION AND DETECTION PROCESSES USING REVERSIBLE AFFINITY ELECTROPHORESIS

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/076,614, filed on March 3, 1998, entitled "Purification and Detection Processes Using Reversible Affinity Electrophoresis," the entire teachings of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Zonal electrophoresis, and particularly gel electrophoresis, is one of the best known methods for separation, purification and characterization of charged molecules, particularly macromolecules such as proteins or nucleic acids (Freifelder, Physical Biochemistry, 2nd ed., (1982) pp. 276-310, Freeman, San Francisco). Electrophoresis can be used to separate molecules based on their size, charge, conformation, and many combinations of these properties.

In most electrophoresis applications, charged molecules migrate through a supporting medium under the influence of an electric field. Most frequently, electrophoresis is carried out using a linear constant voltage gradient of fixed orientation (two fixed electrodes, constant voltage). However, for very large DNA molecules (i.e., in the size range of 30 to 2000 kb), the polymeric chain orients with the field and snakes through the gel rendering the sieving action of the electrophoretic

medium ineffective. In order to separate large DNA molecules, workers have developed applications in which the field orientation is varied cyclically, as in "field inversion gel electrophoresis" (Carle, *et al.*, *Science* (1986), *232*:65) or "pulsed field" gel applications (Schwartz and Cantor, *Cell* (1984), *37*:67). Another technique is to apply a constant field in a cyclic pulsed fashion. Finally, approaches that combine both alternating field and pulsed field duration have been described (Bio-Rad Life Science Research Products Catalog (1997), pp. 175-182).

In most electrophoresis applications, the supporting medium acts to suppress convection and diffusion, and can be sieving or nonsieving. In affinity electrophoresis, the support medium is also modified with chemical groups (i.e., ligands) that interact specifically or nonspecifically with one or more desired analytes and, thus, help to accomplish the separation of analyte and non-analyte sample components during purification by influencing its mobility.

Affinity electrophoresis has been used to measure the binding affinity of proteins (Horejsi and Kocourek, *Biochim. Biophys. Acta* (1974), *336*:338-343 and Chu *et al.*, *J. Med. Chemistry* (1992), *35*:2915-2917). In addition, vinyl-adenine modified polyacrylmide media has been used to enhance resolution of nucleic acids in capillary electrophoresis (Baba *et al.*, *Analytical Chemistry* (1992), *64*:1920-1924).

While many advances have been made in the resolving power of electrophoresis, many biological macromolecules

that contain only slight structural differences, for example, a point mutation in a protein or nucleic acid, still cannot be successfully separated. Analytical techniques that improve resolution of biological molecules are needed to provide researchers with the opportunity to further probe and understand biological systems.

SUMMARY OF THE INVENTION

An affinity electrophoresis process is described, in which the direction of the electric field is varied in a cyclical manner while synchronously changing one, or more, properties of the electrophoretic medium between two states. In the first state, the property or properties which are being varied favor specific reversible binding of sample analytes to affinity ligands which are immobilized within the medium. In the second state, the property or properties which are being varied disfavor the binding of sample analytes to the immobilized affinity ligands. The process provides a convenient method to obtain high resolution separations.

In another embodiment, an apparatus for separating a target analyte from a test sample is described. The apparatus combines an electrophoretic medium having an immobilized affinity ligand, an electrode system having one, or more, electrodes, capable of generating an electric field which can change in orientation, and a means of changing one, or more, properties of the electrophoretic medium between two states. In the first state, the property or properties which are being varied favor

specific reversible binding of sample analytes to affinity ligands which are immobilized within the medium. In the second state, the property or properties which are being varied disfavor the binding of sample analytes to the immobilized affinity ligands. The means of changing a property in the electrophoretic medium can be a device which changes the temperature of the electrophoretic medium. Alternatively, the means of changing one, or more, properties of the electrophoretic medium can be manually or automatically changing the electrophoresis buffer.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B, and 1C show the separation of 5'-Fluorescein-TGA GGC TTT CTG TTA TGG TAC-3' (SEQ ID NO: 1) on an electrophoretic medium having a covalently bound complementary nucleic acid strand from a non-complementary, fluorescently labeled nucleic acid.

Figures 2A, 2B, 2C and 2D show the separation of E. coli Rnase P RNA from 16S Hha RNA and 16S Alu RNA on an electrophoretic medium having a covalently bound nucleic acid sequence that is complementary to a sequence in E. coli Rnase P RNA.

Figures 3A and 3B show the separation of E. coli Rnase P RNA from 16S Hha RNA, 16S Alu RNA and total unlabeled RNA from E. coli on an electrophoretic medium having a covalently bound nucleic acid sequence that is complementary to a sequence in E. coli Rnase P RNA.

DETAILED DESCRIPTION OF THE INVENTION

The invention disclosed herein is directed to an electrophoretic process of separating sample components, and an apparatus designed to carry out the process, that combines the following features: 1) an electrophoretic medium that contains one or more immobilized affinity ligands; 2) use of an electric field that changes in orientation at least once during the process; and 3) a change in at least one other medium property that affects the ability of the affinity ligand(s) to form a specific binding complex with the analyte(s), said change in medium property (or properties) occurring synchronously with the change in field orientation, thereby allowing electrophoretic separation of analyte and non-analyte components of the sample.

The combination of these features results in a novel invention that has broad utility for separation, purification and detection of molecules, including proteins, nucleic acids, and other charged species.

In one preferred embodiment of the invention, the process is a general method for performing repetitive cycles of affinity separation for purification of specific analytes in a biological or test sample. In this embodiment, the analyte molecules are purified (e.g., isolated or separated) from non-analyte sample components. Each cycle is characterized by two electrophoretic steps. The first step is carried out using a first field orientation and first medium condition (also referred to herein as "state"), said condition allowing formation of

specific binding complexes between sample analytes and affinity ligands in the medium. Under those conditions, only non-analyte sample components will migrate in the medium, allowing fractionation of the sample based on analyte affinity for the ligand. The next step is carried out under a second field direction and second medium condition which is obtained by varying one or more property of the electrophoretic medium from the first medium condition. The second condition is designed to disrupt formation of specific binding complexes between sample analytes and affinity ligands in the medium. During this step, all sample components are moved to new locations within the medium. At the completion of this step partial, or complete, separation of specific analytes from other sample components has occurred, and both fractions have been moved to new locations within the medium.

For some sample/analyte/ligand combinations, a single cycle may provide sufficient purification of analyte for many applications. If additional purification is desired, the purification cycle can be repeated. For example, during the second cycle, purification proceeds as in the first cycle. However, the starting materials for the second purification cycle are the partially fractionated products of the first purification cycle which are now at new locations in the electrophoretic medium. The locations of analyte and non-analyte fractions from the first cycle may or may not overlap, depending on the extent of purification achieved in the previous cycle. In either case, during the second and subsequent cycles, the two fractions are further

separated. Thus, an arbitrarily high number of affinity purification cycles can be performed on a single electrophoresis unit, in an automated fashion, with continuous removal of non-binding sample components during each cycle. In systems in which there is tight binding between a gel-immobilized affinity ligand and an analyte and low non-specific binding of non-target molecules for the affinity ligand, a low number of cycles, for example 1 to 10 cycles, may provide the necessary purification. In other cases, where ligand-target binding is weak, or where there is significant binding of non-target molecules to the affinity ligand, many cycles, for example 10 to several thousand, may be required to separate the components.

The repetitive nature of the process allows extremely efficient electrophoretic purification of the analyte molecules. For instance, if the purification efficiency of each cycle is 10-fold (e.g., 90% of non-analyte sample components removed per cycle), four cycles would yield a purification of 10,000-fold.

A key advantage of the invention is that the cyclic purification process can be carried out in a single device, such as an electrophoretic gel. This simplifies the purification process by eliminating preparation, loading, and fraction collection from multiple columns.

In addition, for applications involving simple changes of state, such as temperature, to control ligand/analyte interactions, the process can be performed in an automated fashion using equipment with few (or no) moving parts.

Discussions of the key components of the invention and nonlimiting extensions are listed below:

TEST SAMPLE

The test sample can be any sample, from any source in which analyte molecules are mixed with non-analyte molecules. An analyte molecule is any molecule of interest that can form a binding complex with an affinity ligand. Specifically encompassed by the present invention are samples from biological sources containing cells, obtained using known techniques, from body tissue (e.g., skin, hair, internal organs), or body fluids (e.g., blood, plasma, urine, semen, sweat). Other sources of samples suitable for analysis by the methods of the present invention are microbiological samples, such as viruses, yeasts and bacteria: plasmids, isolated nucleic acids and agricultural sources, such as recombinant plants.

The test sample is treated in such a manner, known to those of skill in the art, so as to render the analyte molecules contained in the test sample available for binding. For example, a cell lysate can be prepared, and the crude cell lysate (e.g., containing the target analyte as well as other cellular components) can be analyzed. Alternatively, the target analyte can be partially isolated (rendering the target analyte substantially free from other cellular components) prior to analysis. Partial isolation can be accomplished using known laboratory techniques. For example, DNA, RNA and proteins can be isolated from a variety of biological samples using TRI reagent (see Sigma catalogue, p. 1545, catalogue numbers T9424, T3809, and T3934, see also Chomczynski, *et al.*, *Biochem.* (1987), *162*:156; Chomczynski, *Biotechniques* (1993), *15*:532) in

conjunct with Southern blotting (DNA), Northern blotting (RNA) and Western blotting (proteins) procedures. Antibodies can be isolated by binding to Protein A immobilized on a solid support (see Surolia, *et al.*, *Trends Bioch. Sci.* (1981), 7:74 and Sigma catalogue p. 1462, catalogue number PURE-1). A nucleic acid analyte can also be amplified (e.g., by polymerase chain reaction or ligase chain reaction techniques) prior to analysis.

AFFINITY LIGANDS THAT BIND REVERSIBLY TO AN ANALYTE

An affinity ligand is any molecule that can form a specific binding complex with an analyte and can be immobilized within a suitable electrophoretic medium.

Methods for determining the thermal stability of binding complexes and, in particular, hybridization complexes are well known in the literature. Wetmur, *Critical Reviews in Biochemistry and Molecular Biology*, 26:227-259 (1991); Quartin and Wetmur, *Biochemistry*, 28:1040-1047 (1989). Application of these methods to estimate the stability of an analyte/affinity ligand complex concerns the following reaction:

$$D + D' \underset{k_r}{\overset{k_2}{\rightleftharpoons}} B \qquad (1)$$

wherein D and D' are an affinity ligand and an analyte, such as a first nucleic acid and a second nucleic acid containing a region complementary to the first nucleic acid

sequence, B is the analyte/affinity ligand complex product and k_2 and k_r are the kinetic rate constants for the analyte/affinity ligand complex formation and dissociation, respectively. In this scheme, the reverse reaction is most relevant to the consideration of spontaneous dissociation of the analyte/affinity ligand complex, and the rate constant for dissociation, k_r, is the critical variable that needs to be minimized to facilitate binding between the analyte and affinity ligand. For a given analyte/affinity ligand complex, dissociation can be reduced by lowering the assay temperature; this will decrease the dissociation constant.

Once a measurement of the dissociation constant has been obtained for one experimental temperature, the Arrhenius equation (2) can be rearranged to calculate the k_r for other temperatures as follows:

$$k = A \exp(-Ea/RT) \qquad (2)$$

$$k_{r1}/k_{r2} = \exp[(Ea/R)\{1/T_2) - (1/T_1)\}] \qquad (3)$$

wherein k_{r1} and k_{r2} are the analyte/affinity ligand complex dissociation rate constants at temperature T_1 and T_2, Ea is the activation energy for dissociation and R is the universal gas constant. For a nucleic acid analyte/affinity ligand complex, the term Ea can be calculated from the base sequence of the nucleic acid sequence used to form the analyte/affinity ligand complex. Wetmur, *Critical Reviews in Biochemistry and Molecular*

Biology, 26:227-259 (1991). Use of the Arrhenius equation for this calculation is described by Tinocco, *et al.*, *Physical Chemistry*: Principles and Applications in Biological Sciences, Prentice Hall (pub.), Englewood Cliffs, NJ, pp. 290-294 (1978).

In the case where the analyte/affinity ligand binding reactions occur in discrete regions of a solid support matrix, such as an electrophoresis matrix, an effective dissociation constant can be estimated using a temperature gradient procedure. The melting behavior of an immobilized analyte/affinity ligand complex within an electrophoresis gel can be measured using a temperature gradient which increases laterally across the gel. The temperature, Td, at which 50% of the complex has dissociated during the time of electrophoresis, ta, can be used to estimate the dissociation constant.

Considering the dissociation as a first order reaction with kinetic rate constant k_r, it follows that at Td:

$$\ln(0.5) = -k_r ta \qquad (4)$$

$$k_r = -0.693/ta \qquad (5)$$

Thus, using temperature gradient gels allows for the measurement of an effective value for k_r, Td and ta. Once k_r has been evaluated at Td, equation (3) can be used to calculate k_r at other lower temperatures that might be suitable for the first medium condition wherein conditions are selected to allow the formation of specific binding

complexes between sample analytes and affinity ligands. These calculated values of k_r can then be used with the first order rate law to calculate the fraction of analyte/affinity ligand complex remaining at a given assay temperature ta and electrophoresis time ta:

$$\ln(B/Bo) = -k_r ta \quad (6)$$

wherein B is the concentration of analyte/affinity ligand complex remaining at time ta, and Bo is the initial concentration of the complex. Equation (6) can be used to estimate the change in k_r needed to increase B/Bo (decrease analyte/affinity ligand complex dissociation) by any specified amount. Once the desired value of k_r is known, equation (3) can be used to calculate the change in temperature needed to achieve the k_r value.

It should be noted that the gradient gel procedure only provides an estimate of the actual analyte/affinity ligand complex Td and k_r, since displaced analytes can rebind to uncomplexed immobilized affinity ligands. In general, the experimentally determined values will overestimate the actual Td and underestimate the actual k_r for the reversible analyte/affinity ligand complex dissociation reactions. Nevertheless, the quantitative relationships given in equations (1) through (6) provide a rational and practical framework for predicting the stability of analyte/affinity ligand complexes, and design of medium conditions for separation protocols.

One especially useful example of an affinity ligand is

a single-stranded nucleic acid, which can bind by hybridization, for example, to an analyte that contains a complementary nucleic acid sequence. The single strand nucleic acid affinity ligand can be complementary to the entire analyte nucleic acid sequence or to a portion thereof. Single-stranded nucleic acids can also be used for isolation of duplex nucleic acids by triplex formation (Hogan and Kessler, U.S. Patent No. 5,176,966 and Cantor, *et al.*, U.S. Patent No. 5,482,836, the teachings of which are incorporated herein by reference). Double-stranded nucleic acids can also serve as useful affinity ligands for nucleic acid binding proteins, or for nucleic acid analytes that bind to the ligand by triplex or tetraplex formation.

The conditions under which a single strand nucleic acid will bind to another nucleic acid to be immobilized in a gel can be estimated using the procedure outlined above for estimating the stability of analyte/affinity ligand complexes. In addition, the melting temperature (Tm) of the two nucleic acids provides a reasonable framework for estimating the temperate at which an nucleic acid analyte will hybridize to a nucleic acid affinity ligand. In general, the Td is lower than the Tm by about 15 to 25°C and, therefore, the temperature at which the gel should be run to facilitate specific hybridization between the analyte and affinity ligand should be about 15 to 25°C or more below the Tm.

The Tm of a pair of nucleic acids is typically determined by monitoring a physical property, such as UV absorption, of a solution of the two nucleic acids in the

electrophoresis buffer while uniformly varying the property of the solution that will be cyclically varied during the electrophoresis separation. For example, the temperature can be slowly decreased while monitoring the UV absorption. At high temperatures the nucleic acids are single stranded. As the temperature decreases complementary bases pair off and hydrogen bond. This hydrogen bonding causes a change in UV absorption. If the nucleic acids are complementary, the transition between the hydrogen bonding state and the non-hydrogen bonding state occurs over a narrow temperature range. The midpoint of this temperature range is the Tm for the two nucleic acids. Similarly, the ionic strength or pH of the buffer can be varied in a uniform manner while holding the temperature constant and monitoring the UV absorption.

Nucleic acids form duplexes more readily in higher ionic strength and lower temperature conditions. "Stringency conditions" for hybridization is a term of art which refers to the conditions of temperature and buffer concentration (ionic strength) which permit hybridization of a particular nucleic acid to a second nucleic acid in which the first nucleic acid may be perfectly complementary to the second, or the first and second may share some degree of complementarity which is less than perfect. For example, certain high stringency conditions can be used which distinguish perfectly complementary nucleic acids from those of less complementarity. "High stringency conditions" and "moderate stringency conditions" for nucleic acid hybridizations are explained on pages 2.10.1-

2.10.16 (see particularly 2.10.8-11) and pages 6.3.1-6 in *Current Protocols in Molecular Biology* (Ausubel, F.M. *et al.*, eds., Vol. 1, containing supplements up through Supplement 29, 1995), the teachings of which are hereby incorporated by reference. The exact conditions which determine the stringency of hybridization depend not only on ionic strength, temperature and the concentration of destabilizing agents such as formamide, but also on factors such as the length of the nucleic acid sequence, base composition, percent mismatch between hybridizing sequences and the frequency of occurrence of subsets of that sequence within other non-identical sequences. Thus, high or moderate stringency conditions can be determined empirically.

By varying hybridization conditions from a level of stringency at which no hybridization occurs to a level at which hybridization is first observed, conditions which will allow a given sequence to hybridize (e.g., selectively) with the most similar sequences in the sample can be determined. Binding conditions for triplexes and tetraplexes can be estimated in a similar manner.

Nucleic acid aptamers (Tuerk and Gold, *Science* (1990) *249*:5050; Joyce, *Gene* (1989), *82*:83-87; Ellington and Szostak, *Nature* (1990), *346*:818-822) can also be used as affinity ligands in the process of the present invention. Aptamers can be selected against many kinds of analytes, including proteins, small organic molecules, and carbohydrates (reviewed in Klug and Famulok, *Molecular Biology Reports* (1994), *20*:97-107). Thus, selection of

aptamer ligands offers a simple and flexible mechanism for obtaining affinity ligands against virtually any target molecule.

Other useful ligands include proteins or polypeptides which can bind to specific analytes. An especially useful class of protein ligands are antibody molecules, which can be elicited against a wide range of analytes by immunization methods. Antibodies ligands can be monoclonal or polyclonal. In addition, a fragment of an antibody can be an affinity ligand. Similarly, receptor proteins may be useful as ligands for purification and detection of analytes that bind to or are bound by them.

Carbohydrates have been successfully used as affinity ligands for electrophoretic purification of lectins (Horejsi and Kocourek, *Biochim. Biophys. Acta* (1974), *336*:338-343), and may be useful for purification and detection of molecules that bind to specific carbohydrates or glycoproteins.

Binding or non-binding conditions of proteins, aptamers and lectins for specific ligands can be estimated using the procedure outlined above for estimating the stability of analyte/affinity ligand complexes. In addition, equilibrium dialysis experiments can provide a rational method of predicting the stability of analyte/affinity ligand complexes. For example, the dissociation constant of a protein for a particular ligand can be determined in the electrophoresis buffer at several different pHs, temperatures or ionic strengths. The higher the dissociation constant, the weaker the binding between

the protein and the ligand (see Segel, I.H., *Biochemical Calculations, 2nd Edition* (1976), John Wiley & Sons, N.Y., p. 241-244). From this data a binding and a non-binding condition can be estimated.

Many other types of immobilized ligands are possible including peptides, amino acids, nucleosides, small organic molecules, lipids, hormones, drugs, enzyme substrates, enzyme inhibitors, enzymes, coenzymes, inorganic molecules, chelating agents, macromolecular complexes, polysaccharides, monosaccharides, and others.

AN ELECTROPHORETIC MEDIUM WHICH CONTAINS AT LEAST ONE IMMOBILIZED AFFINITY LIGAND

Any medium suitable for electrophoresis can be used for the methods of the present invention. In general, suitable media fall into two classes. The first includes media composed of gel-forming materials like crosslinked polyacrylamide and agarose. The second class includes media composed of solutions of linear noncrosslinked polymers such as polyacrylamide, poly(hydroxyethylcellulose), and poly(ethyleneoxide). The latter category is commonly used for capillary electrophoresis applications.

Immobilization of ligands can be accomplished by direct attachment to the polymeric components of the medium. Such attachment can be mediated by formation of covalent bonds between the ligand and the polymer. Noncovalent binding between the ligand and polymer substituents can also be used. For instance, strong

noncovalent binding provided by the widely-used biotinstreptavidin and digoxigenin-antidigoxigenin systems can be used to attach ligands to appropriately modified polymeric media. Covalent attachment is preferred.

Direct connection between the polymeric medium and the ligand is not strictly required. For instance, ligands can be attached to particulate supports, such as microspheres, and the particulate supports can be immobilized within the polymer medium by physical entrapment (Cantor, *et al.*, U.S. Patent No. 5,482,863, the teachings of which are incorporated herein by reference in their entirety). The particles may be macroscopic, microscopic, or colloidal in nature, (see Polyciences, Inc., 1995-1996 particle Catalog, Warrington, PA).

In a similar manner, ligands can be attached to highly branched soluble polymers. Due to their branched shape, such ligand-polymer complexes display extremely large effective hydrodynamic radii and, therefore, will not migrate in the electric field in many kinds of polymeric media of appropriately small pore size. Thus, they can be entrapped within the media in the same fashion as particulate supports.

Absolute immobilization of the ligand within the medium is not required for all embodiments of the invention. For many applications, it is sufficient that the mobility of the analyte is changed upon formation of a binding complex with the ligand. This condition can be satisfied by coupling the ligand to a medium component that has extremely low electrophoretic mobility. However, for

efficient purification the change in mobility should be as large as possible. Therefore, media utilizing true immobilization of the ligand within the medium will be preferred for use in this invention.

Commonly used gel media useful for the present invention include acrylamide and agarose gels. However, other materials may be used. Examples include modified acrylamides and acrylate esters (for examples see Polysciences, Inc., Polymer & Monomer catalog, 1996-1997, Warrington, PA), starch (Smithies, *Biochem. J.* (1959), *71*:585; product number S5651, Sigma Chemical Co., St. Louis, MO), dextrans (for examples see Polysciences, Inc., Polymer & Monomer Catalog, 1996-1997, Warrington, PA), and cellulose-based polymers (for examples see Quesada, *Current Opinions in Biotechnology* (1997), *8*:82-93). Any of these polymers can be chemically modified to allow specific attachment of ligands (including nucleic acids, proteins, peptides, organic small molecules, and others) for use in the present invention.

For some methods, it may be useful to use composite media, containing a mixture of two or more supporting materials. An example is the composite acrylamide-agarose gel. These gels typically contain from 2-5% acrylamide and 0.5%-1% agarose. In these gels the acrylamide provides the chief sieving function, but without the agarose, such low concentration acrylamide gels lack mechanical strength for convenient handling. The agarose provides mechanical support without significantly altering the sieving properties of the acrylamide. In such cases, the nucleic

acid can be attached to the component that confers the sieving function of the gel, since that component makes most intimate contacts with the solution phase nucleic acid target.

For capillary electrophoresis (CE) applications it is convenient to use media containing soluble polymers. Examples of soluble polymers that have proven to be useful for CE analyses are linear polymers of polyacrylamide, poly(N,N-dimethylacrylamide), poly(hydroxyethylcellulose), poly(ethyleneoxide) and poly(vinylalcohol) as described in Quesada, *Current Opinion in Biotechnology* (1997), *8*:82-93). Solutions of these polymers can also be used to practice the methods of the present invention.

Methods of coupling a variety of ligands to create affinity electrophoresis media are well known to those skilled in the art. Many ligands can be coupled to agarose, dextrans, cellulose, and starch polymers using cyanogen bromide or cyanuric chloride activation. Polymers containing carboxyl groups can be coupled to ligands that have primary amine groups using carbodiimide coupling. Polymers carrying primary amines can be coupled to aminecontaining ligands with glutaraldehyde or cyanuric chloride. Many polymers can be modified with thiol-reactive groups which can be coupled to thiol-containing ligands. Many other suitable methods are known in the literature. For examples, see Wong, "Chemistry of Protein Conjugation and Cross-linking", CRC Press, Boca Raton, FL, 1993.

Methods for covalently attaching ligands by copolymerization with the polymeric material of the electrophoretic medium have also been developed. In this approach, ligands are chemically modified with a copolymerizable group. When such modified ligands are copolymerized with suitable mixtures of polymerizable monomers, polymeric media containing high concentrations of immobilized ligand can be produced. Examples of methods for covalently attaching nucleic acids to polymerizable chemical groups are found in U.S. Patent Application Serial No. 08/812,105, entitled "Nucleic Acid-Containing Polymerizable Complex," and U.S. Patent Application Serial No. 08/971,845, entitled "Electrophoretic Analysis of Molecules Using Immobilized Probes," the teachings of which are herein incorporated by reference, in their entirety. (See also, Rehman, *et al.*, *Nucleic Acids Research* (1999), *27*:649.) Other useful methods that have been used to immobilize proteins and small organic molecules within polymer layers and gels are described in Bille *et al.*, *Eur. J. Biochem.* (1989), *180*:41-47; Wang *et al.*, *Nature Biotechnology* (1997), *15*:789-793; and Holtz and Asher, *Nature* (1997), *389*:829-832.

Other approaches for attaching nucleic acid probes to preformed polyacrylamide polymers, including gels or linear soluble polymers can be found in Ghosh and Fahy, U.S. Patent No. 5,478,893, the teachings of which are incorporated herein by reference in their entirety, and in Timofeev *et al.*, *Nucleic Acids Res.* (1996), *24*:3142-3148.

PROCESS AND MEANS FOR VARYING ORIENTATION OF ELECTROPHORETIC FIELD DURING SEPARATION

An electrode system is a system that, in conjunct with a power supply, produces and electric field gradient. An electric field gradient is the voltage drop across the gel created by the electrode system (see Giddings, Unified Separation Science (1991), John Wiley & Sons, New York, p. 155-170). The orientation of the electric field gradient used for electrophoresis determines the geometry of the separation between analyte and non-analyte sample components. Many field geometries can be used. For instance, with a conventional two-electrode apparatus, a one-dimensional separation can be achieved simply by switching the polarity of the two electrodes, as practiced in field inversion gel electrophoresis (Carle *et al.*, *Science* (1986), *232*:65). In separations of this sort, analytes only migrate under conditions which disfavor binding to the ligands, whereas the non-analyte sample components would migrate under both sets of conditions. For purposes of illustration, let the direction of net analyte movement during the purification process be called "forward". If the purification cycle is designed so that the duration of reverse field orientation is longer than the duration of forward field orientation, analyte molecules will be moved forward during each purification cycle, but non-analyte sample components will only enter the gel only transiently since they are efficiently removed from the gel by the long period of reverse field orientation.

Two dimensional electrode arrangements, as used in pulsed field (Schwartz and Cantor, *Cell* (1984), 37:67) and CHEF applications (CHEF gels, U.S. Patent No. 5,549,796; Bio-Rad Life Science Research Products Catalog (1997), pp. 175-182), allow the separation process of the present invention to be performed in two dimensions. In principle, the addition of another set of electrodes operating in a third dimension could add additional separation capability if desired.

The state of instrumentation and methodology for performing one and two dimensional electrophoretic separations is well advanced. At least one commercially available device (CHEF gel apparatus, Bio-Rad Life Science Research Products Catalog, 1997, pp. 175-182), offers the capability of performing two-dimensional electrophoretic separations with programmable automated control of field orientation and pulse duration.

PROCESS AND MEANS FOR VARYING AT LEAST ONE OTHER PROPERTY OF THE ELECTROPHORETIC MEDIUM IN SYNCHRONY WITH THE CHANGE IN ELECTROPHORETIC FIELD ORIENTATION

The electrophoretic medium can be reversibly cycled between at least two different user-defined states by varying one or more property of the electrophoretic medium (e.g., temperature, pH or ionic strength) . In one state, the ligand has a relatively high affinity for the analyte of interest. In the other state, the ligand has relatively low binding affinity with the analyte. In a preferred embodiment, non-analyte sample components have low affinity

for the ligand in both states. In a more preferred embodiment of the invention, variation in medium state and the orientation of the electric field are co-regulated, so that electrophoresis of non-analyte materials occurs under all conditions and field orientations, but electrophoresis of analytes occurs only under a limited set of conditions and field orientations. Thus, analytes and non-analyte molecules will have different net mobilities for each cycle, and their separation in the medium will increase with each cycle.

Changing the medium temperature is one preferred means for modulating analyte-ligand binding affinity, since temperature can be varied with little or no manipulation of the electrophoresis medium, and since a great deal of instrumentation for temperature control is commercially available. However, other medium properties may be used as well. A non-limiting list of possible properties which are known to affect noncovalent chemical associations include changes in medium pH, changes in the ionic strength of the medium, and other changes in chemical composition of medium.

In one especially preferred embodiment of the invention, the affinity ligand is an nucleic acid and the analyte is a sample nucleic acid that has at least one region complementary to the affinity ligand nucleic acid. In this case, the binding between analyte and ligand can be effectively modulated by changing the gel temperature. For example, at temperatures above the Td of the ligand-analyte complex, binding affinity will be low. Similarly, at

temperatures below the Td, binding affinity will be substantially higher.

Processes and means for cycling electrophoretic media between two temperatures are well known to those skilled in the art. For example, temperature- controlled equipment for performing vertical or horizontal format electrophoresis are commercially available (Bio-Rad Life Science Research Products Catalog (1997), pp. 127-133, 175-182; Pharmacia Biotech BioDirectory (1997), pp. 345, 309, 334). In some instruments, temperature control is achieved by circulation of water (or suitable coolant) through the instrument. In these instruments, temperature cycling can be achieved by the switching coolant source between two regulated reservoirs set at the desired temperatures. In some electrophoresis instruments, the medium is in thermal contact with a programmable thermocycler which relies on the Petier effect for heating and cooling. (Thermocyclers can be obtained from MJ Research, Watertown MA). For example, and electrophoresis unit with a Peltier heating/cooling devise can be obtained from Pharmacia (Pharmacia Biotech BioDirectory (1997), pp. 334).

Another method of modulating the analyte-ligand binding affinity is by changing the ionic strength of the electrophoresis buffer. The ionic strength of the buffer that will facilitate binding is dependent on the type of analyte and the affinity ligand. In general, a buffer that has a higher ionic strength facilitates binding. Buffers that have ionic strengths of about 100 mM to 1 M are

preferred during the state in which the analyte is bound to the affinity ligand. Buffers that have ionic strengths of about 10 mM or less are preferred during the state in which the analyte is not bound to the affinity ligand. Equilibrium dialysis or hybridization experiments can be used to provide a rational for predicting the stability of a particular analyte/affinity ligand binding complex at a particular ionic strength.

Another method of modulating the analyte-ligand binding affinity is by changing the electrophoresis buffer to a denaturing buffer. A denaturing buffer contains chemicals (hereinafter "denaturants") which disrupt the binding of the analyte to the affinity ligand. For example, formamide or urea can be a component of the denaturing buffer. The amount of denaturant required will depend on the type of target molecule, the type of affinity binding interaction, field strength, ionic strength, and temperature of electrophoresis. In general, the denaturing buffer can have a very broad concentration range of formamide or urea. Formamide can be used in concentrations up to 95% (volume/volume), and urea can be used at concentrations up to 8M. Equilibrium dialysis or hybridization experiments can also be used to provide a rational for predicting the stability of the analyte/affinity ligand binding complex in a particular denaturing buffer.

In some instances, the analyte-ligand binding affinity can also be modulated by changing the pH of the buffer. For example, nucleic acids will hybridize to a

complementary nucleic acid affinity ligand at or near neutral pH (e.g., pH 6-8). DNA affinity ligands hybridized to DNA targets can be disrupted by acidic pH (e.g., below pH 5) or basic pH (e.g., above pH 11). Changing the pH of the electrophoresis buffer is a preferred method of disrupting the binding of protein analytes to an affinity ligand. Equilibrium dialysis experiments can be used to estimate the pH range for binding and dissociation of a particular protein to an analyte.

For maximum separation efficiency, switching medium conditions should switch the analyte between completely bound and completely unbound states. This clean distinction between bound and unbound analyte states can be achieved with single-stranded nucleic acid analyte-ligand systems, as exemplified in later sections of this disclosure. However, such absolute two-state behavior is not required for successful application of the invention. In general, it is sufficient that the mobility of the analyte be substantially altered by the change in medium conditions. Here "substantially" means that the mobility change observed in a ligand-containing medium is greater than the mobility change observed for a similar medium lacking the ligand, or alternatively, a medium containing a ligand which is chemically similar to the original ligand but which is incapable of forming specific binding complexes with analyte. Given such a substantial change in analyte mobility, even weak specific ligand/analyte interactions can be used successfully to practice the present invention, since an arbitrarily large number of

purification cycles can be repeated in an automated fashion. In these cases, each cycle gives a small but finite separation between analyte and non-analyte sample components, and the purification cycle is repeated until the required level of separation is achieved.

APPLICATIONS OF THE INVENTION

The invention can be used to purify analytes for subsequent characterization and other preparative purposes. In addition, the invention can be used for detection of analytes.

For preparative purposes, the invention is powerful because it allows the potential for performing many repetitive cycles of affinity purification using a single automated programmable device with inexpensive, easy to prepare affinity media. Preferably, elution of purified product could be accomplished electrophoretically using a variation of methods disclosed in Gombocz *et al.* U.S. Patent No. 5,217,591 and Kragt and Ballen, U.S. Patent No. 3,989,612, the teachings of which are incorporated herein by reference in their entirety. Other elution methods are possible and are well known to those skilled in the art.

For detection purposes, the invention is powerful because it allows removal of non-analyte sample components which can contribute to unfavorable levels of background. The general format of these assays involves the following steps:

1) preparation of sample to be tested;

2) purification of potential sample analyte by

method of present invention; and

3) assay for analyte in output of purification process in step 2.

As nonlimiting examples of useful detection applications using the invention, three variations of this general scheme are presented below. The examples are not intended to limit the scope of the invention in any way. Other detection methods may be more useful for other types of analyte. All are directed toward detection of bacteria in a blood sample.

a) Detection by dye binding

In step 1, total RNA is prepared from the blood sample (Chomczynski, et al., *Anal. Biochem.* (1987), *162*:156; Chomczynski, *Biotechniques* (1993), *15*:532; TRI Reagent BD, Sigma (1999), catalog no. T3809, p. 1545). In step 2, RNA is subjected to the method of the present invention using an affinity medium specific for binding bacterial ribosomal RNA. In step three, the output of the purification process is tested for the presence of RNA using dyes which fluoresce brightly when bound to nucleic acids, for example, Acridine Orange, SYBR green II, TO-TO or YO-YO. Other examples of suitable dyes can be found in Haugland, "*Handbook of Fluorescent Probes and Research Chemicals, 6th Edition,*" Molecular Probes, Eugene, OR, (1996), pp. 144-156.

b) Detection by copurification of enzymatic label

In step 1, total RNA is prepared from the blood sample, and the bacterial ribosomal RNA analyte within the sample is bound to an enzymatic reporter molecule, such as alkaline phosphatase, horseradish peroxidase, or luciferase by means which are not disrupted during the purification process of step 2. Preferably, attachment of the RNA analyte to the enzyme reporter is mediated by a nucleic acid sandwich hybridization probe (Ranki and Soderland, U.S. Patent No. 4,486,539; Engelhardt and Rabbani, U.S. Patent No. 5,288,609, the teachings of which are incorporated herein by reference in their entirety) to which the enzyme is conjugated. The assay of step 3 detects the enzymatic reporter molecule using chromogenic, chemifluorescent, or chemiluminescent substrates.

c) Detection by copurification of amplifiable reporter label

In step 1, total RNA is prepared from the blood sample, and the bacterial ribosomal RNA analyte within the sample is bound to an amplifiable reporter molecule, such as a substrate of Q-beta replicase, for example the MDV-1 substrate of Q-beta replicase (Lizardi *et al.* Bio/Technology 6:1197-1202, 1988), by means which are not disrupted during the purification process of step 2. Preferably, attachment of the RNA analyte to the enzyme reporter is mediated by a nucleic acid sandwich hybridization probe, to which the amplifiable reporter is conjugated. The assay of step 3 detects the amplifiable

reporter molecule using an appropriate enzymatic amplification and detection process.

It will be obvious to those skilled in the art that the examples (a) through (c) above are listed in order of increasing sensitivity. The theoretical sensitivity of example (c) is limited only by loss of analyte and/or amplifiable reporter during sample preparation and electrophoretic purification, since the Q-beta replicase system is capable of detecting a single MDV-1 substrate in a sample.

The invention is illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLES

Example 1. Separation of nucleic acid samples

An example of the present invention used to separate a mixture of nucleic acids, is shown in Figure 1A and 1B. In this example, the ligand is an nucleic acid GTA CCA TAA CAG CAA GCC TCA (SEQ ID NO: 2) covalently immobilized in an standard polyacrylamide gel using the methods of U.S. patent applications Serial Nos. 08/812,105 and 08/971,845, the teachings of which are incorporated herein by reference in their entirety.

A fluorescently labeled nucleic acid, 5'-Fluorescein-ATT ACG TTG ATA TTG CTG ATT A-3' (SEQ ID NO: 3), that is not complementary to the ligand (signified by a box) was loaded in lane 1. A fluorescently labeled nucleic acid, 5'-Fluorescein-TGA GGC TTT CTG TTA TGG TAC-3' (SEQ ID

NO: 1), complementary to the ligand was loaded in lane 2. This preparation contains a minor species (signified by an open circle) which shows a slightly greater mobility than the major species (signified by closed circles) for unknown reasons. Lane 3 contained a mixture of the two fluorescein-tagged nucleic acids.

Figures 1A and 1B show the gel after the end of steps 1 and 2 in the first cycle. In step 1, the gel was maintained at 45°C. At this temperature, binding of the ligand to complementary nucleic acids was prevented, and the electric field, applied for 43 minutes at 100 V, had the polarity indicated in Figure 1. In step two, the gel was maintained at 25°C, a temperature which allows binding of the ligand to complementary nucleic acids. The electric field, applied for 50 min at 100 V, had the opposite polarity to that used in step 1. At the end of step one, the mobility of the two fluorescently labeled nucleic acids was similar, as shown in Figure 1A. At the end of step two, a separation of the two nucleic acids was observed (Figure 1B). Figure 1C, shows the same gel after a total of three cycles. Each cycle consisted of steps 1 and 2 (down/hot and up/cold) as described for Figures 1A and 1B, above. After the third cycle, the separation between the complementary and noncomplementary nucleic acids was increased. In particular, the complementary nucleic acid moved further down the gel, while the noncomplementary nucleic acid moved in the reverse direction.

GENERATION OF RNA ANALYTES USED IN EXAMPLES 2 AND 3

Three RNA analytes were used for the experiments shown in Figures 2 and 3. All three were generated by in vitro transcription reactions using T7 polymerase (enzyme from Boehringer Mannheim catalog #881,767; other reaction components from Promega kit catalog #P1420) with fluorescently tagged ribonucleotide triphosphates as label (fluorescein labeled nucleotides from Boehringer Mannheim catalog #1,685,61 9). The three RNA molecules were:

1. 16S Hha, 502 nucleotides in length, nonspecific sample component.
2. 16S Alu, 255 nucleotides in length, nonspecific sample component.
3. E. coli RNase P RNA, 377 nucleotides in length, specific RNA analyte.

These RNA species were generated as detailed below:

16S Hha and 16S Alu

Plasmid pSCH038 was obtained from the American Type Culture Collection (ATCC87435). The plasmid is a PCRII plasmid vector (Invitrogen, Carlsbad, California, catalogue number K2050-01) containing the E. coli 16S ribosomal RNA (rRNA) sequence from positions 674 to 1411. The complete 16S molecule is 1541 nucleotides in length. The vector contains a promoter site for T7 RNA polymerase to the 5' side of insert so that in vitro transcription with that enzyme will produce RNA with the same sense as native 16S rRNA.

Two 5 mg aliquots of plasmid were digested with the restriction enzymes Hha I or Alu I. The digested plasmid samples were used as templates for in vitro transcription with T7 polymerase and fluorescein-labeled nucleotides. After synthesis the DNA templates were removed by digestion with Dnase I, and the transcripts were purified from unincorporated nucleotides using Pharmacia G25 spin columns.

The first 68 nucleotides of both transcripts are derived from the cloning vector PCRII: there are 68 nucleotides between the T7 initiation site and the 16S insert. Alu I cleaves between nucleotides 860 and 861 of the 16S rRNA sequence. Therefore, the length of the transcript generated from the Alu 1 cleaved template, 16S Alu, is 255 nucleotides.

Hha I cleaves the 16S sequence between nucleotides 1107 and 1108 of the 16S rRNA sequence. Therefore, the length of the 16S Hha transcript is 502 nucleotides.

E. coli Rnase P RNA

Naturally occurring E. coli Rnase P RNA is 377 nucleotides in length (Altman, *et al.*, *In tRNA: Structure, Biosynthesis, and Function* (1995), p. 67-78, editors Soll and RajBhandary, American Society of Microbiology, Washington, D.C.). A PCR fragment containing this sequence was generated from E. coli genomic DNA. The amplification primer on the 5' side of the gene contained a T7 RNA polymerase promoter. Thus, the resulting 396 bp DNA amplification product included a T7 promoter immediately 5'

of the Rnase P RNA gene sequence. This DNA was used as a template in in vitro transcription reactions using T7 polymerase to generate fluorescently-labeled Rnase P RNA, as described above. The in vitro transcript produced from this template was identical in sequence to the published sequence of E. coli Rnase P RNA.

Example 2. Separation of E. coli Rnase P RNA from 16S Hha RNA and 16S Alu (see Figures 2A, 2B, 2C and 2D)

In this example, the ligand is an nucleic acid 5'-CCA TCG GCG GTT TGC TCT CTG TTG-3' (SEQ ID NO: 4) covalently immobilized in an standard polyacrylamide gel using the methods of U.S. patent applications Serial Nos. 08/812,105 and 08/971,845. This ligand is complementary to a sequence contained within E. coli Rnase P RNA. The ligand was attached via its 5' terminus, and was present in the gel at a concentration of 10 mM (strands). The gel contained 5% polyacrylamide (29:1, monomer:bisacrylamide), 45 mM Tris-Borate pH 8.3, and 2 mM EDTA (0.5XTBE buffer). The gel was run in a temperature-controlled minigel apparatus (Penguin vertical gel box, Owl Scientific, Woburn, MA). Gel temperature was controlled by pumped water from external recirculating water baths. For all electrophoresis steps, the applied field was 200 volts.

The left lane was loaded with fluorescein-labeled E. coli Rnase P RNA. The right lane was loaded with a mixture of all three fluorescein-labeled transcripts, Rnase P, 16S Hha, and 16S Alu.

The Figure 2A shows the pattern of fluorescent products after electrophoresis down through the gel for 10 minutes at 54°C, a gel temperature which should disrupt binding of the gel ligand to the specific analyte, Rnase P RNA. The gel was imaged directly without removing the glass plates using a Molecular Dynamics Fluorimager. The gel was replaced in the gel box and equilibrated at 41°C, a temperature which should allow hybridization of the nucleic acid ligand to the Rnase P RNA. The gel ligand is not complementary to the 16S transcripts. At this temperature, the samples were electrophoresed upward for 20 minutes. The field was shut off and the gel temperature was changed to 59°C. The samples were then electrophoresed down for 5 minutes, and a new gel image of the samples was taken, as shown in Figure 2B. At this point, only the Rnase P RNA analytes remain in the gel, demonstrating the efficient removal of the 16S transcripts during the upward electrophoresis step. The gel was returned to the apparatus and electrophoresed up at 41°C for 10 minutes and down at 59°C for 10 minutes. Another gel image was taken as shown in Figure 2C. The gel was then electrophoresed down at 59°C for 25 minutes, and a final image was obtained as shown in Figure 2D. The Rnase P RNA samples migrate progressively down the gel as shown in Figures 2C and 2D, demonstrating release of the specific RNA analytes during the high temperature downward electrophoresis steps.

Example 3. Separation of E. coli Rnase P RNA from 16S Hha RNA, 16S Alu RNA and total unlabeled RNA from E. coli (see Figures 3A and 3B)

The ligand-containing gel was prepared as in Example 2. Lane 1 contained E. coli Rnase P RNA; lane 2, 16S Alu transcript; lane 3, 16S Hha and 16S Alu; lane 4, 16S Hha and Rnase P RNA; lane 5, 6.3 mg total unlabeled RNA from E. coli; lane 6, Rnase P RNA, 16S Hha, 16S Alu, and 6.3 mg total unlabeled RNA from E. coli. The gel was equilibrated to 50°C, a temperature which should disrupt hybridization of ligand to the specific analyte, Rnase P RNA, and samples were electrophoresed down for 10 minutes. An image of the gel after step 1 is seen in Figure 3A. The gel was equilibrated at 41°C and electrophoresed up for 40 minutes. At this temperature, the Rnase P RNA can hybridize with the gel ligand. An image of the gel after step 2, shown in Figure 3B, demonstrates the complete removal of the 16S Hha and Alu transcripts from the gel. The specific retention of Rnase P RNA analyte in the gel was not affected by an excess of heterogeneous unlabeled RNA in lane 6, further demonstrating the high specificity of analyte capture.

EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled

in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

CLAIMS

What is claimed is:

1. A method of separating an analyte from other components in a test sample comprising the steps of:
   a) contacting an electrophoretic medium having an immobilized affinity ligand with the test sample under conditions wherein the affinity ligand binds selectively to the analyte, referred to as the initial conditions;
   b) applying an electric field oriented in a first direction for a period of time sufficient to cause the migration of non-analyte material in the test sample; and
   c) changing the direction of the electric field while simultaneously varying at least one property of the electrophoretic medium, wherein said variation reduces the binding affinity of the affinity ligand for the analyte sufficient to allow the analyte to migrate in the electric field,

   thereby separating the analyte from other components in the test sample.

2. The method of Claim 1, further comprising the step of returning to the initial conditions and repeating step b) followed by repetition of step c) one or more times.

3. The method of Claim 2, wherein the orientation of the electric field can be varied in one dimension.

4. The method of Claim 2, wherein the orientation of the electric field can be varied in two dimensions.

5. The method of Claim 2, wherein the orientation of the electric field can be varied in three dimensions.

6. The method of Claim 1, wherein the property of the electrophoretic medium that is varied is temperature.

7. The method of Claim 1, wherein the property of the electrophoretic medium that is varied is the chemical composition.

8. The method of Claim 7, wherein the chemical composition of the electrophoretic medium is varied by replacing the electrophoresis buffer.

9. The method of Claim 7, wherein the variation in chemical composition results in a variation in pH of the electrophoretic medium.

10. The method of Claim 7, wherein the chemical composition is varied by varying the concentration of at least one denaturant.

11. The method of Claim 10, wherein the denaturant is formamide.

12. The method of Claim 10, wherein the denaturant is urea.

13. The method of Claim 7, wherein the variation in chemical composition results in a variation in ionic strength of the electrophoretic medium.

14. The method of Claim 1, wherein the affinity ligand is selected from the group consisting of: a peptide, amino acid, nucleoside, nucleotide, nucleic acids, small organic molecule, lipid, hormone, drug, enzyme substrate, enzyme inhibitor, enzyme, coenzyme, inorganic molecule, chelating agent, macromolecular complex, polysaccharide, or monosaccharide.

15. The method of Claim 1, wherein the affinity ligand is a single-stranded nucleic acid sequence.

16. The method of Claim 15, wherein the analyte is an nucleic acid, wherein a portion of the nucleic acid analyte is complementary to a portion of the nucleic acid affinity ligand.

17. The method of Claim 16, wherein the initial conditions are high stringency hybridization conditions.

18. The method of Claim 16, wherein the initial conditions are medium stringency hybridization conditions.

19. The method of Claim 1, wherein the affinity ligand is a double-stranded nucleic acid sequence.

20. The method of Claim 1, wherein the affinity ligand is an aptamer.

21. The method of Claim 1, wherein the affinity ligand is a protein.

22. The method of Claim 21, wherein the affinity ligand is an antibody.

23. The method of Claim 1, wherein the affinity ligand is a carbohydrate.

24. The method of Claim 1, wherein the analyte is bound to a fluorescent dye before contacting the analyte with the electrophoretic medium.

25. The method of Claim 1, wherein the analyte is bound to an enzymatic reporter molecule before contacting the analyte with the electrophoretic medium.

26. The method of Claim 19, wherein the enzymatic reporter molecule is alkaline phosphatase.

27. The method of Claim 1, wherein the analyte is bound to an amplifiable reporter molecule before contacting the analyte with the electrophoretic medium.

28. The method of Claim 27, wherein the amplifiable reporter molecule is a substrate of Q-beta replicase.

29. An apparatus for carrying out the method of Claim 1, comprising:

a) an electrophoretic medium having an immobilized affinity ligand;

b) a power supply capable of generating an electric field sufficient to cause migration of components in the test sample;

c) at least one electrode system connected to the power supply and the electrophoretic medium which orients the electric field gradient, wherein the orientation of the electric field gradient of each electrosystem is reversible;

d) a first buffer reservoir in contact with the electrophoretic medium;

e) a second buffer reservoir in contact with the electrophoretic medium; and

f) a means of varying at least one property of the electrophoretic medium.

30. The apparatus of Claim 29, wherein the orientation of the electric field can be varied in one dimension.

31. The apparatus of Claim 29, wherein the orientation of the electric field can be varied in two dimensions.

32. The apparatus of Claim 29, wherein the orientation of the electric field can be varied in three dimensions.

33. The apparatus of Claim 29, wherein the property of the electrophoretic medium that is varied is the chemical composition.

34. The apparatus of Claim 33, wherein the chemical composition of the electrophoretic medium is varied by replacing the electrophoresis buffer.

35. The apparatus of Claim 33, wherein the variation in chemical composition results in a variation in pH of the electrophoretic medium.

36. The apparatus of Claim 33, wherein the chemical composition is varied by varying the concentration of at least one denaturant.

37. The apparatus of Claim 36, wherein the denaturant is formamide.

38. The apparatus of Claim 36, wherein the denaturant is urea.

39. The apparatus of Claim 33, wherein the variation in chemical composition results in a variation in ionic strength of the electrophoretic medium.

40. The apparatus of Claim 29, wherein the property of the electrophoretic medium which is varied is the temperature.

41. The apparatus of Claim 40, wherein the temperature is varied by circulation of a fluid from at least one temperature-regulated reservoir.

42. The apparatus of Claim 40, wherein the temperature is varied by a Peltier-effect heating/cooling device.

43. The apparatus of Claim 40, wherein the orientation of the field gradient and the variation in temperature are controlled in an automated programable fashion.

44. The apparatus of Claim 40, wherein the temperature is varied by increasing the voltage produced by the power supply.

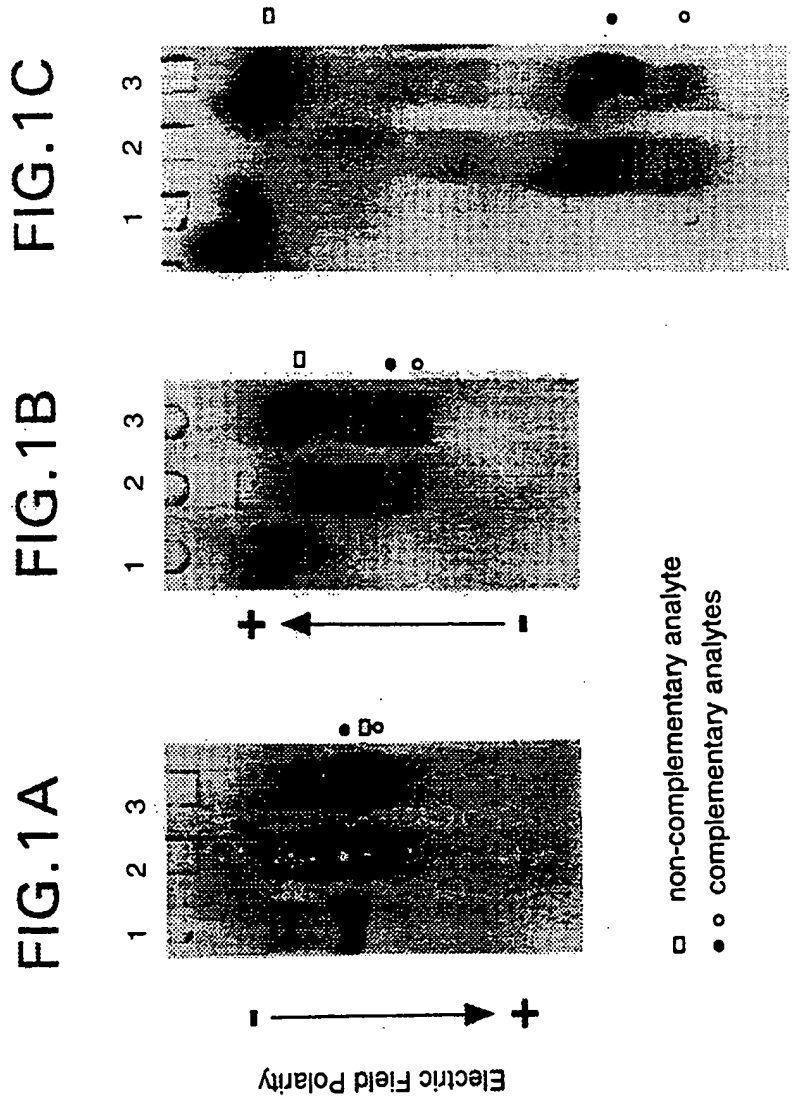
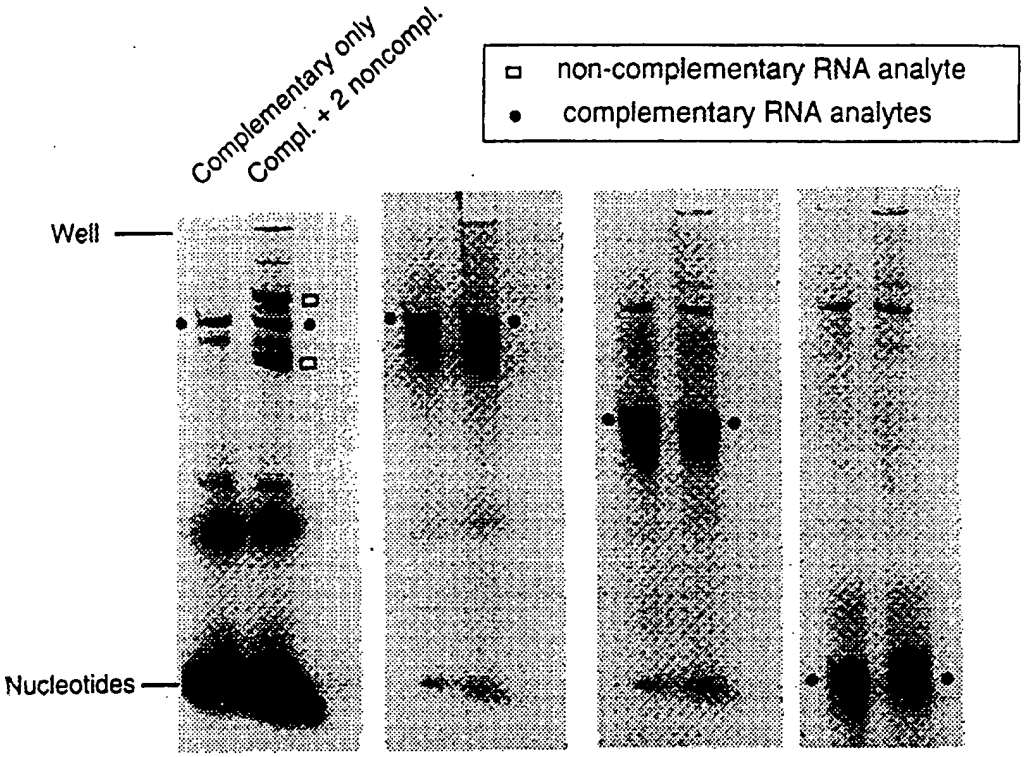


FIG.1C
FIG.1B
FIG.1A
1 2 3
+
Electric Field Polarity
non-complementary analyte
complementary analytes



HOT,10 min.

FIG.2A

Cold,20 min.
HOT,5 min.

FIG.2B

Cold,10 min.
HOT,10 min.

FIG.2C

HOT,25 min.

FIG.2D

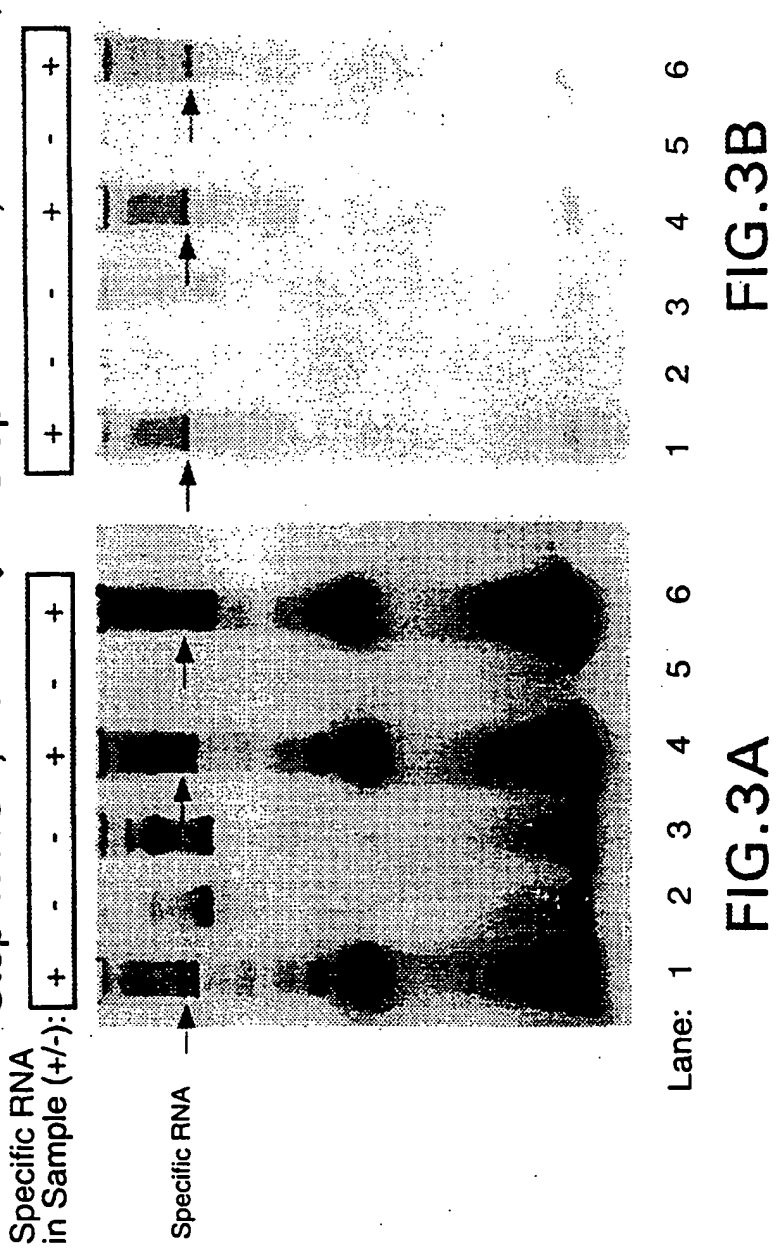

Step 1. HOT, 10 min.
Step 2. COLD, 40 min.
Specific RNA in Sample (+/-):
+ - - + - +
+ - - + - +
Specific RNA
Lane: 1 2 3 4 5 6
1 2 3 4 5 6

FIG.3A

FIG.3B